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Elevated TLR5 expression *in vivo* and loss of NF-κB activation via TLR5 *in vitro* detected in HPV-negative oropharyngeal squamous cell carcinoma

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Abstract

In oropharyngeal squamous cell carcinoma (OPSCC), the expression pattern of toll-like receptors (TLRs), in comparison between human papillomavirus (HPV)-positive and -negative tumors differs. TLRs control innate immune responses by activating, among others, the nuclear factor-κB (NF-κB) signaling pathway. Elevated NF-κB activity is detectable in several cancers and regulates cancer development and progression. We studied TLR5 expression in 143 unselected consecutive OPSCC tumors, and its relation to HPV-DNA and p16 status, clinicopathological parameters, and patient outcome, and studied TLR5 stimulation and consecutive NF-κB cascade activation *in vitro* in two human OPSCC cell lines and immortalized human keratinocytes (HaCat). Clinicopathological data came from hospital registries, and TLR5 immunoexpression was evaluated by immunohistochemistry. Flagellin served to stimulate TLR5 in cultured cells, followed by analysis of the activity of the NF-κB signaling cascade with In-Cell Western for IκB and p-IκB.

High TLR5 expression was associated with poor disease-specific survival in HPV-positive OPSCC, which typically shows low TLR5 immunoexpression. High TLR5 immunoexpression was more common in HPV-negative OPSCC, known for its less-favorable prognosis. *In vitro*, we detected NF-κB cascade activation in the HPV-positive OPSCC cell line and in HaCat cells, but not in the HPV-negative OPSCC cell line.

Our results suggest that elevated TLR5 immunoexpression may be related to reduced NF-κB activity in HPV-negative OPSCC. The possible prognosis-worsening mechanisms among these high-risk OPSCC patients however, require further evaluation.

Keywords

Oropharyngeal squamous cell carcinoma, human papillomavirus, toll-like receptor, Nuclear factor-κB

Abbreviations

DSS	Disease-specific survival
DAMP	danger-associated molecular pattern
HPV	Human papillomavirus
HNSCC	Head and neck squamous cell carcinoma
IKK	IκB kinase
p-IκB	phosphorylated form of IκB protein
NF-κB	Nuclear factor-κB
OPSCC	Oropharyngeal squamous cell carcinoma
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptors
SCC	Squamous cell carcinoma
TLR	Toll-like receptor
TMA	Tissue microarray
TNM	Tumor lymph node metastasis classification

Introduction

According to the latest WHO classification of head and neck tumors, the oropharyngeal squamous cell carcinoma (OPSCC) that is associated with human papillomavirus (HPV) and the HPV-unrelated OPSCC are distinct entities (El-Naggar et al., 2017). Incidence of HPV-positive OPSCC is increasing (Nichols et al., 2013; Rietbergen et al., 2013; Chaturvedi et al., 2011; Ang et al., 2010; Nasman et al., 2009; Gillison et al., 2000), whereas incidence of HPV-negative OPSCC has remained stable or has declined (Chaturvedi et al., 2011; Nasman et al., 2009). Patients with HPV-positive OPSCC have a more favorable prognosis than do those with OPSCC that is HPV-negative (Ang et al., 2010; Gillison et al., 2000). Differences between these two OPSCC have been under intense study for their underlying mechanisms involved in carcinogenesis and tumor behavior, and ultimately to allow the design of targeted OPSCC-prevention, -diagnostic, and -treatment strategies.

Toll-like receptors (TLRs) participate in the initiation of innate immune cascades by recognizing pathogen-associated molecular patterns (PAMPs) of bacteria, viruses, fungi, and parasites, and endogenous danger-associated molecular patterns (DAMPs) in cells that are damaged or dying (Kawai and Akira 2005; Matzinger 2002; Janeway 1989). Activation of TLRs may promote carcinogenesis, yet other reports suggest anti-tumor responses and a role in halting tumor progression (Basith et al., 2012; Ioannou and Voulgarelis 2010). TLRs may be prognostic in several cancers including colorectal-, salivary gland-, and squamous cell carcinoma of the tongue and non-small cell lung cancer (Makinen et al., 2015; Korvala et al., 2014; Zhou et al., 2014; Kauppila et al., 2013; Grimm et al., 2010). In OPSCC, expression patterns of TLR5, 7, and 9 between HPV-positive and HPV-negative tumors differ (Jouhi et al., 2015). Recently, prognostic values have been proposed for TLR5 and 7 in HPV-positive OPSCC (Jouhi et al., 2017).

TLR activation by PAMPs or by DAMPs leads to activation of nuclear factor-κB (NF-κB), a key transcription factor involved in the inflammatory pathway. NF-κB integrates multiple stress stimuli and regulates innate and adaptive immune responses during inflammation and during cancer development and progression (Karin et al., 2002; Balkwill and Mantovani 2001). It influences cancer initiation, promotion, and progression by enhancing cancer cell proliferation (Luo et al., 2004), preventing apoptosis (Van Antwerp et al., 1996), and by improving metastatic potential (Huber et al., 2004) and angiogenesis (Balkwill and Mantovani 2001). Elevated NF-κB

activity is detectable in breast cancer (Chua et al., 2007), melanoma (Yang et al., 2007), colon cancer (Scartozzi et al., 2007), multiple myeloma (Annunziata et al., 2007), pancreatic cancer (Weichert et al., 2007), and lung cancer (Tew et al., 2008).

Inactive NF-κB is localized in the cytosol, complexed with an inhibitory protein belonging to the IκB family (Baeuerle and Baltimore 1988b; Baeuerle and Baltimore 1988a). Initiation of NF-κB activation occurs by degradation of the IκB protein of the NF-κB complex by IκB kinase (IKK) (Beg et al., 1993; Shirakawa and Mizel 1989). IκB-protein degradation leads to activation of NF-κB and release of a short-lived phosphorylated form of IκB protein (p-IκB) that will eventually be degraded in the proteasome (Beg et al., 1993). Activated NF-κB then enters the nucleus, initiating gene activation as a fast response to a stimulus such as TLR activation by PAMP or DAMP (Baeuerle and Baltimore 1988b; Baeuerle and Baltimore 1988a). According to Ludde *et al.* (2007), elimination of NF-κB activity in mouse hepatocytes resulted in elevated inflammatory cytokine expression and spontaneous carcinogenesis suggesting a tumor suppressor role for NF-κB (Luedde et al., 2007). Liu *et al.* (2006), on the other hand, observed reduced immunoexpression of IKK-α as paralleling the increasing aggressiveness of human skin squamous cell carcinoma (SCC) when evaluated by SCC grade (Liu et al., 2006). Maeda *et al.* saw IKK-α to be less strongly expressed in oral carcinomas than in normal oral epithelium (Maeda et al., 2007).

We aimed to investigate TLR5 expression, its relation to HPV-DNA and p16 status, clinical parameters, and patient outcome *in vivo* in 143 unselected consecutive OPSCC patients. Furthermore, we studied flagellin-induced TLR5 stimulation and consecutive NF-κB-cascade activation *in vitro* in two human OPSCC lines and in immortalized human keratinocytes.

Materials and methods

Patients and clinicopathological data

The patient cohort, as described in part elsewhere (Carpen et al., 2018; Carpen et al., 2017), included 143 of 224 consecutive patients diagnosed with a primary OPSCC between February 2012 and May 2016 at Helsinki University Hospital, Finland. Inclusion criteria for the study were available tumor HPV-DNA PCR and $p16^{INK4a}$(p16) status, and availability as a tissue microarray (TMA) block. Excluded were patients with palliative treatment. Clinicopathological data: age, sex, tumor histology, grade, UICC 8th edition TNM stage (Brierley et al., 2017), primary treatment, and patient outcome and status at last follow-up, we manually recorded from hospital registries. Patients were in two categories: having treatment by surgery followed by chemoradiotherapy called Sx + C(RT) or having only chemoradiotherapy C(RT). Their follow-up scheme has been described earlier (Carpen et al., 2018). This study received the approval of the Research Ethics Board of the Hospital District of Helsinki and Uusimaa, with institutional permission granted.

For evaluation purposes, the data classification was as proposed by Smeets et al. into an HPV-positive group of 89 that included only p16-positive and HPV-DNA-positive samples, and an HPV-negative group that included 18 p16-positive but HPV-DNA-negative samples, 33 p16-negative HPV-DNA-negative samples, and 3 p16-negative but HPV-DNA-positive samples (Table 1) (Smeets et al., 2007). The data reflect the current reported characteristics for HPV-related and non-related OPSCC according to the latest TNM 8 classification (Nauta et al., 2018; Gillison 2016; Gillison et al., 2008).

Immunohistochemistry

Tissue-microarray (TMA) -blocks were prepared and slides were stained by immunohistochemistry as described (Makinen et al., 2012). For expression of TLR5 in the tumor TMA samples, the immunohistochemical staining was done with monoclonal mouse anti-human TLR5 IgG (1:100, NBP2-24787, Novus Biologicals, Littleton, CO, USA).

Immunoscore

Two of the authors (J.H. and A.K.K.) scored independently the decoded TMA blocks immunostained with the TLR5 antibody. Any discordance in scoring was solved by reassessment to achieve consensus. TLR scoring was based on intensity of positivity in the tumor tissue as described (Jouhi et al., 2015). Scoring of TLR5, present on the plasma membrane and in the cytoplasm, was on the scale: none (0), mild (1), moderate (2), strong (3). If several tumor spots were available for analysis, our choice was the highest TLR5 positivity value.

Cell culture

Cell-culture experiments involved two human OPSCC lines UT-SCC-65 and UT-SCC-69 (Department of Otorhinolaryngology-Head and Neck Surgery, University of Turku, Finland), and the immortalized human keratinocyte cell line HaCaT. Earlier we had evaluated p16 immunoexpression and HPV-DNA PCR targeting the E6/E7 region from pelleted UT-SCC-65 and UT-SCC-69 cells (Jouhi et al., 2015). In the UT-SCC-65 cell line, p16 status was negative, but HPV-DNA status was positive. In UT-SCC 69, both p16 status and HPV-DNA status were positive. However, a recent study investigated HPV-DNA in UT-SCC-65 by PCR and described it as HPV negative (Lepikhova et al. 2018). Thus, based on an algorithm suggested by Smeets et al. (Smeets et al., 2007), and findings of Lepikhova et al. (Lepikhova et al., 2018), we considered UT-SCC-65 to be HPV negative.

The UT-SCC cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 4.5g/l glucose, supplemented with 10% fetal calf serum (FCS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), non-essential amino acids (NEAA), glutamine, penicillin, and streptomycin. The HaCat cells were cultured at 37°C in DMEM medium containing 1.0g/l glucose with 5% FCS, penicillin, streptomycin, glutamine, and sodium pyruvate. NEAA and sodium pyruvate were from Gibco (Thermo Fisher Scientific Inc.,

Waltham, MA, USA), glutamine from Lonza (Lonza Group AG, Basel, Switzerland), and all other cell culture reagents from Sigma-Aldrich, (Merck KGaA, Darmstadt, Germany).

In-Cell Western

Cells on 96-well plates were stimulated with 100 ng/ml flagellin (FLA) (InvivoGen, San Diego, CA, USA) for 15-120 minutes. Thereafter, the cells were fixed with 2% paraformaldehyde (PFA) for 20 min, and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were treated with Odyssey Blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 30 min, followed by incubation with mouse-anti phospho-IκB (p-IκB) IgG (Cell Signaling Technology, Leiden, Netherlands, dilution 1:200) or rabbit-anti IκB IgG (Cell Signaling Technology, dilution 1:50). Subsequently, the cells were incubated with IRDye 800 donkey anti-mouse or anti-rabbit IgGs and nuclear marker DRAQ-5 (Thermo Fisher Scientific), which served for normalization. Detection and quantification of the signal was performed on three parallel samples with Odyssey Infrared Imager and software (LI-COR Biosciences). The experiment was repeated three times and the results were calculated as an average of the three parallel samples in the three experiments.

Statistical analysis

SPSS version 25.0 (IBM SPSS Statistics, IBM Corporation, New York, NY, USA) was utilized for statistical analysis. The cross-tabulation of categorical variables was performed using Chi-square with an asymptotic or an exact *P*-value when most suitable. Disease-specific survival (DSS) rates were calculated by Kaplan-Meier (KM) estimate. Evaluation of statistical significance in the KM analysis utilized log-rank test. Follow-up time in the DSS was defined as the period between the last treatment day and the last day of follow-up, or date of death from the disease. The Cox proportional hazards model served in univariate and multivariable survival analysis. The analysis included clinically relevant variables with a *P*-value less than 0.1. The proportional hazard

assumption was tested with KM curves. An independent t-test served for comparison of mean values of continuous data between categorical groups with two classes.

The relative signal values in the cell culture experiments are presented as mean ±SD. Students t-test (Microsoft Inc., Redmond, WA, USA) served to determine the significance of the relative signal of IκB and p-IκB at measuring points (15, 30, 60, and 120 min). A two-sided *P*-value less than 0.05 was considered statistically significant.

Results

TLR5 immunoexpression and its association with patient and tumor characteristics

TLR5 was expressed in 94 (65.7%) of the OPSCC and was associated with HPV status (*P*=0.018) (Table 1). High TLR5 immunoexpression more often occurred in the HPV-negative OPSCC.

TLR5 was associated with N class, tumor site, and tumor grade (Table 2). The proportion of N0-grade OPSCC among patients with high TLR5 immunoexpression was higher (*P*=0.008). Regardless of TLR5 immunoexpression level, the tonsils dominated as the primary tumor site. Proportionally higher TLR5 immunoexpression was also related to a primary tumor site other than the tonsils, namely the base of the tongue, the soft palate, or the posterior wall of the oropharynx (*P*=0.006). Proportionally higher TLR5 immunoexpression was associated with grade-1 and -2 tumors than with grade-3 tumors (*P*=0.004).

TLR5 immunoexpression and its association with survival

For the survival analysis, TLR5 immunoexpression was dichotomized into low (immunoscore 0-1) and high (immunoscore 2-3). Patients with high TLR5 immunoexpression had less favorable DSS (79.1%) than did those

with low TLR5 immunoscores (94.7%) (*P*=0.006). In HPV-positive OPSCC that typically shows low TLR5 immunoexpression, patients with high TLR5 immunoexpression had poorer DSS (85.3%) than did those with low TLR5 immunoexpression, at 98.2%, (*P*=0.024). The HPV-negative subgroup showed no statistically significant association between DSS and TLR5 immunoexpression (Figure 1).

TLR5 as an independent prognostic factor in HPV-positive subgroups

In the multivariable analysis of all 143 patients, TLR5 immunoexpression was not an independent prognostic factor (*P*=0.141) (Table 3). Although TLR5 had no significant interaction with HPV status (*P*=0.116), stratifying the analysis according to HPV status was considered clinically justified.

Among HPV-positive OPSCC patients, high TLR5 immunoexpression was an independent prognostic factor for poor DSS (*P*=0.028). No other factors showed independent statistical significance for DSS among HPV-positive patients (Table 3). Among HPV-negative patients, no factors independently influenced the DSS.

NF-κB cascade activation *in vitro* by stimulation of TLR5

The results of our *in vivo* studies on the role of TLR5 in OPSCC led us to study the possible mechanisms involved *in vitro*. In cell culture experiments, we stimulated the TLR5 receptor with flagellin and measured the expression level of the negative regulator of the NF-κB signaling cascade, namely Inhibitor of the κB (IκB) and its inactive phosphorylated form IκB (p-IκB). NF-κB activation is preceded by a phosphorylation of IκB to p-IκB (Beg et al., 1993; Shirakawa and Mizel 1989), giving us the means to qualitatively evaluate TLR5 activation of the NF-κB signaling cascade by quantitative changes in IκB and in p-IκB expressions.

The expression of TLR5, NF-κB, IκB, and p-IκB *in vitro* were qualitatively confirmable in the immunoblotting experiments in all cell lines: HPV-negative OPSCC line UT-SCC-65, HPV-positive OPSCC line UT-SCC-69,

and HaCat cells (data not shown). The TLR5 stimulation with flagellin led to a decreased amount of IκB in HaCaT cells during the first 30 minutes (at 0, and at 15-, and 30-min timepoints). At the 60-min and 120-min timepoints, IκB then increased, exceeding the amount measured at 0 minutes. In the HPV-positive OPSCC cell line UT-SCC-69, IκB decreased during the first 60 minutes of incubation (at 0, 15-, 30-, and 60-minute timepoints) and returned to its initial level in 120 minutes. No statistically significant decrease in IκB was detectable in the stimulated HPV-negative OPSCC cell line UT-SCC-65 cultivation at any of the timepoints during the 120-minute follow-up (Figure 2A). No significant changes occurred in the amount of p-IκB following flagellin stimulation in any of the cell lines studied (Figure 2B). In the HaCat cells and in the HPV-positive OPSCC cell line UT-SCC-69 the amount of p-IκB relative to IκB (p-IκB/ IκB) increased during the first 30 and 60 minutes of incubation, respectively, and then returned to its initial level or below in 120 minutes. In HPV-negative OPSCC cell line UT-SCC-65 there was no statistically significant change in the relative amount of p-IκB/ IκB during the 120-minute follow-up (Figure 2C).

Discussion

This study shows that TLR5 stimulation with flagellin did not activate NF-κB signaling in HPV-negative OPSCC, that typically has high TLR5 expression. In addition, this study provides further evidence for TLR5 expression as being an independent prognostic biomarker in HPV-positive OPSCC. Our results from multivariable analysis indicate that TLR5 expression was not an independent prognostic factor among all 143 patients. However, high TLR5 expression was an independent indicator of poor DSS in those with HPV-positive OPSCC. The association of high TLR5 expression and poor DSS among HPV-positive OPSCC patients validates our earlier observation for the p16-positive and the HPV-DNA-positive patients (Jouhi et al., 2017). As in our earlier retrospective patient series, TLR5 immunoexpression appeared not to be prognostic in HPV-negative OPSCC (Jouhi et al., 2017).

TLRs, having both tumorigenic and antitumorigenic effects, participate in immunomodulation of the tumor microenvironment. Activation of the TLRs by PAMPs derived from microbes, and by DAMPs derived from injured and necrotic cancer cells, leads to a release of cytokines and chemokines by the activated cells. TLRs expressed in the immune cells contribute to chronic inflammation and an attack against tumor cells, whereas TLRs expressed during carcinogenesis by tumor cells are proposed to promote cancer-cell survival and chemoresistance (Basith et al., 2012). A worsening effect of high TLR5 expression on prognosis is supported by the finding of increased TLR5 expression in association with a higher mortality rate and disease recurrence in oral mobile-tongue squamous cell carcinoma (SCC) (Kauppila et al., 2013). A similar association also occurs for progression of cervical neoplasia and carcinogenesis of the stomach and colon (Pimentel-Nunes et al., 2013; Pimentel-Nunes et al., 2012; DeCarlo et al., 2012; Kim et al., 2008). Contradictory findings also exist: low TLR5 expression predicting a poor prognosis in oral mobile-tongue squamous cell carcinoma (SCC) (Makinen et al., 2015), whereas high expression of TLR5 correlates with poor prognosis in non-small cell lung cancer (Zhou et al., 2014).

High TLR5 immunoexpression was more common in the HPV-negative OPSCC. Our earlier study showed that strong *Treponema denticola*-specific protease (*Td*-CTLP) immunoexpression was associated with high TLR5 expression and low TLR 7 expression in the HPV-DNA-negative OPSCC, and that strong *Td*-CTLP immunoexpression was associated with a poor DSS. Interestingly, strong immunoexpression of *Td*-CTLP occurred mainly among the HPV-DNA-negative tumors (Kylma et al., 2018). This led us to study the prognostic role of TLR5 in the current patient series and its possible functional role *in vitro* involved with HPV-positive and –negative OPSCC cell lines.

In cell culture experiments on HPV-positive and HPV-negative OPSCC cell lines, we studied phosphorylation of IκB to p-IκB followed by TLR5 stimulation with flagellin, to evaluate the level of NF-κB signaling cascade activation. Typically, p-IκB expression is higher after treatment of cells with various inducers, and then rapidly decreases following its degradation in a proteasome (Beg et al., 1993). Re-accumulation of the inactive NF-κB-IκB complex in the cytoplasm then follows, because NF-κB directly stimulates IκBa gene expression (de Martin

et al., 1993). We did not detect any difference in the total amount of p-IκB in any of the cell lines studied after stimulation with flagellin. However, we observed an increase in the ratio of p-IκB to IκB at early time points in the HaCat cells and in the HPV-positive OPSCC cell line UT-SCC-69 following stimulation of TLR5 with flagellin. This indicates activation of the NF-κB cascade. The amount of p-IκB to IκB then returned to its initial level in a 120-minute incubation, which may be related to IκBa gene expression as has been proposed by de Martin *et al.* (de Martin et al., 1993). As there were no statistically significant changes in the amount of p-IκB to IκB in the HPV-negative OPSCC cell line UT-SCC-65, we propose that the NF-κB-cascade was not activated by TLR5 stimulation with flagellin in this cell line.

Our earlier study has shown that TLR5 expression level is higher in the HPV-negative cell line UT-SCC-65, than in the HPV-positive cell line UT-SCC-69 reflecting the *in vivo* TLR5 immunoexpression data (Jouhi et al., 2015).

Based on our results, it is impossible to deduce whether any relationship exists between elevated TLR5 expression and failure to activate NF-κB cascade in the HPV-negative OPSCC cell line. Further studies with HPV positive and negative OPSCC cell lines with both low and high level of TLR5 expression could give more information on this matter. However, in an earlier study on Salmonella flagellin in intestinal epithelial cells, Tallant *et al.* (2004) showed that among several cancerous and non-cancerous cell lines, some had an abundance of TLR5 protein and yet showed poor or no NF-κB activation by flagellin. They proposed that this may be due to intracellular location of TLR5, TLR5 gene mutation, or lack of required TLR5 co-receptor or adaptor (Tallant et al., 2004). Earlier, we detected TLR5 immunoexpression both in plasma membrane and in cytoplasm in OPSCC. The localization was independent of HPV status proposing that the localization may not be the cause of missing NF-κB cascade activation by flagellin (Jouhi et al., 2015). Higher TLR5 expression and loss of NF-κB cascade activation by TLR5 stimulation may, on the other hand, be linked to generally poorer patient outcome in HPV-negative OPSCC. This is supported by the observation that NF-κB cascade activation prevents the development of hepatocellular carcinoma in mouse hepatocytes (Luedde et al., 2007).

Recently, evidence shows that those patients with HPV-positive OPSCC and intratumoral HPV-specific type I–polarized T cells have an improved response to standard therapy (Welters et al., 2018). Other research has detected a high level of CD8+ tumor-infiltrating lymphocytes in the HPV-positive OPSCC with low TLR5 immunoexpression (Haeggblom et al., 2019). Furthermore, in a mouse model, stimulation of the TLR5 by flagellin had a radioprotective effect on the non-tumor cells, and yet did not influence tumor-cell radiosensitivity (Burdelya et al., 2008). We may hypothesize that the cytokine response to TLR5 stimulation by flagellin in the HPV-positive versus the HPV-negative OPSCC differs. We may also speculate that TLR5 stimulation of the HPV-negative OPSCC leads to activation of a signaling pathway other than the NF-κB pathway. Tan *et al.* on the other hand, have discussed cross-talk between several pattern recognition receptors (PRRs) sharing overlapping ligand specificities, and immune processes requiring activation of several PRRs to achieve non-additive responses (Tan et al., 2014). It may be, that in addition to TLR5, activation of another PRR is required for effective activation of NF-κB cascade in the HPV-negative OPSCC.

In the HPV-positive cell line and in HaCat cells, that serve in our study as a model for non-cancerous epithelial cells, the NF-κB cascade was activated by stimulation of TLR5 with flagellin. It is well known that HPV-positive OPSCCs have better prognosis than HPV-negative OPSCCs, and thus it is interesting that HPV-positive cell line behaves more like non-cancerous keratinocytes when stimulated with flagellin. This suggests that NF-κB activation may contribute to the more favorable prognosis of HPV-positive OPSCC patients, which is further supported by the observation that TLR5 activation suppresses tumor cell proliferation and progression in human breast cancer (Cai et al., 2011). Yet, another study failed to link activation of TLR5 by flagellin to tumor progression in an oral SCC cell line showing strong expression of TLR5, 7, and 9 (Park et al., 2010). An adverse effect from TLR5 activation has also been detectable in salivary gland adenocarcinoma and in gastric carcinoma, in both of which such activation caused cancer progression (Park et al., 2011; Song et al., 2011).

We consider that our results are applicable to OPSCC patients outside the current patient series because the clinicopathological characteristics of our patients with either an HPV-positive or HPV-negative tumor are in line with those reported for HPV-positive and HPV-negative OPSCC (Nauta et al., 2018; Gillison 2016; Gillison et

al., 2008). However, as patients with HPV-positive OPSCC typically have a better prognosis, the low number of events reduced the power of our survival analysis, as we have suggested earlier (Haeggblom et al., 2019; Jouhi et al., 2017). In addition, expression levels of immune cells or cytokines were not investigated here and call for further investigation.

We studied TLR5 immunoexpression and patient outcome *in vivo* and TLR5 stimulation response *in vitro*, which limits the interpretation of our results to these factors only. The association of high TLR5 expression and poor DSS among HPV-positive patients validates our earlier result (Jouhi et al., 2017), but does not claim any causality between them. However, our findings lead us to propose that HPV-positive and HPV-negative OPSCC cell lines differ in both their TLR5 expression level and in their TLR5 stimulation response, and that these differences may be linked to OPSCC prognosis.

Conclusions

Our results provide further evidence that high TLR5 expression may serve as a prognostic factor for poor disease-specific survival (DSS) in HPV-positive OPSCC. This finding is consistent with our previous findings (Jouhi et al., 2017). Furthermore, *in vitro* stimulation of TLR5 with flagellin activated the NF-κB cascade in HPV-positive OPSCC cells and in HaCat cells, whereas no activation of the NF-κB cascade in HPV-negative OPSCC cells occurred. Elevated TLR5 immunoexpression in HPV-negative OPSCC may be related to missing activation of NF-κB cascade, and the possible prognosis-worsening mechanisms among these high-risk OPSCC patients poses challenges for further evaluation.

Conflict of interest statement

The authors declare no conflict of interest.

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Table 1 Patient and tumor characteristics; age, sex, smoking and alcohol consumption, T class (primary tumor size), N class (presence of regional lymph node metastasis), tumor stage, tumor site of origin, grade of differentiation, treatment (Sx + (C)RT = surgery + chemoradiotherapy, C(RT) = chemoradiotherapy), toll-like receptor (TLR) 5 immunoexpression stratified according to human papilloma virus (HPV) status.

N = 143	HPV-positive *) (n = 89)	HPV-negative **) (n = 54)	*P*	Missing
Age at diagnosis	60.8 (mean)	62.2 (mean)		
Sex				
Male	73 (82.0)	37 (68.5)		
Female	16 (18.0)	17 (31.5)	0.063	0
Smoking				
Never	32 (36.0)	9 (16.7)		
Earlier	37 (41.6)	7 (13.0)		
Current	20 (22.5)	38 (70.4)	**< 0.001**	0
Excess alcohol consumption				
Never	49 (72.1)	21 (41.2)		
Earlier	6 (8.8)	10 (19.6)		
Currently	13 (19.1)	20 (39.2)	**0.003**	24
T class				
T1-T2	58 (65.2)	36 (66.7)		
T3-T4	31 (34.8)	18 (33.3)	0.855	0
N class				
N0	9 (10.1)	16 (29.6)		
N1-N3	80 (89.9)	38 (70.4)	**0.004**	0
Tumor stage				
I-II	72 (80.9)	27 (50.0)		
III-IV	17 (19.1)	27 (50.0)	**< 0.001**	0
Tumor site				
Tonsil	67 (75.3)	22 (40.7)		
Base of tongue	21 (23.6)	14 (25.9)		
Soft palate	1 (1.1)	13 (24.1)		
Posterior wall of oropharynx	0 (0)	5 (9.3)	**< 0.001**	0
Tumor grade				
1	0 (0)	3 (5.6)		
2	7 (7.9)	18 (33.3)		
3	82 (92.1)	33 (61.1)	**< 0.001**	0
Treatment				
Sx ± (C)RT	33 (37.1)	21 (38.9)		
(C)RT	56 (62.9)	33 (61.1)	0.829	0
TLR 5				
0	36 (40.4)	13 (24.1)		
1	19 (21.3)	8 (14.8)		
2	17 (19.1)	10 (18.5)		
3	17 (19.1)	23 (42.6)	**0.018**	0

Statistically significant *P* values bolded.

***)** HPV-positive = p16- and HPV-DNA-positive OPSCC

**) HPV-negative = p16-negative and HPV-DNA-positive or –negative OPSCC, and p16-positive and HPV-DNA negative OPSCC

Table 2 Patient and tumor characteristics (as in Table 1) association with toll-like receptor (TLR) 5 immunoexpression.

	TLR 5					
N = 143	**0 (n = 49)**	**1 (n = 27)**	**2 (n = 27)**	**3 (n = 40)**	***P***	**Missing**
Sex						
Male	41 (83.7)	17 (63.0)	22 (81.5)	30 (75.0)		
Female	8 (16.3)	10 (37.0)	5 (18.5)	10 (25.0)	0.202	0
Smoking						
Never	17 (34.7)	7 (25.9)	7 (25.9)	10 (25.0)		
Earlier	18 (36.7)	9 (33.3)	9 (33.3)	8 (20.0)		
Current	14 (28.6)	11 (40.7)	11 (40.7)	22 (55.0)	0.327	0
Excess alcohol consumption						
Never	25 (65.8)	14 (73.7)	12 (50.0)	19 (50.0)		
Earlier	6 (15.8)	0 (0)	5 (20.8)	5 (13.2)		
Currently	7 (18.4)	5 (26.3)	7 (29.2)	14 (36.8)	0.246	24
T class						
T1-T2	33 (67.3)	20 (74.1)	20 (74.1)	21 (52.5)		
T3-T4	16 (32.7)	7 (25.9)	7 (25.9)	19 (47.5)	0.184	0
N class						
N0	3 (6.1)	3 (11.1)	6 (22.2)	13 (32.5)		
N1-N3	46 (93.9)	24 (88.9)	21 (77.8)	27 (67.5)	**0.008**	0
Tumor stage						
I-II	37 (75.5)	21 (77.8)	20 (74.1)	21 (52.5)		
III-IV	12 (24.5)	6 (22.2)	7 (25.9)	19 (47.5)	0.061	0
Tumor site						
Tonsil	35 (71.4)	21 (77.8)	14 (51.9)	19 (47.5)		
Base of tongue	12 (24.5)	6 (22.2)	9 (33.3)	8 (20.0)		
Soft palate	1 (2.0)	0 (0)	3 (11.1)	10 (25.0)		
Posterior wall of oropharynx	1 (2.0)	0 (0)	1 (3.7)	3 (7.5)	**0.006**	0
Tumor grade						
1	0 (0)	0 (0)	1 (3.7)	2 (5.0)		
2	2 (4.1)	4 (14.8)	5 (18.5)	14 (35.0)		
3	47 (95.9)	23 (85.2)	21 (77.8)	24 (60.0)	**0.004**	0
Treatment						
Sx ± (C)RT	22 (44.9)	11 (40.7)	9 (33.3)	12 (30.0)		
(C)RT	27 (55.1)	16 (59.3)	18 (66.7)	28 (70.0)	0.491	0

Statistically significant *P* values bolded.

Table 3. Univariate and multivariable Cox regression analysis for Disease specific survival (DSS) in a series of all 143 oropharyngeal squamous cell carcinoma (OPSCC) patients, including 89 human papilloma virus (HPV) -positive and 54 HPV-negative OPSCC patients.

	Univariate analysis All patients (N = 143)			Multivariabe analysis All patients (N =143)			Multivariable analysis HPV-positive *) (n = 89)			Multivariable analysis HPV-negative **) (n = 54)		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Sex												
Male vs female	1.3	0.5 - 3.7	0.596									
Age at time of diagnosis, mean	1.1	1.0 - 1.1	**0.013**	1.0	1.0 - 1.1	**0.046**	1.1	1.0 - 1.2	0.165	1.1	1.0 - 1.1	0.118
Smoking			0.060			0.394			0.269			0.622
Earlier vs never	0.5	0.1 - 2.5	0.376	0.7	0.1 - 4.1	0.705	0.9	0.1 - 6.7	0.945	0.0		0.982
Currently vs never	2.3	0.8 - 7.2	0.143	2.0	0.6- 7.2	0.294	5.1	0.5 - 49.	0.160	2.3	0.4 11.7	0.330
T class												
T3-4 vs T1-2	2.0	0.8 - 4.9	0.153									
N class												
N1-3 vs N0	3.6	0.5 - 26.9	0.215									
Tumor stage												
III-IV vs I-II	3.8	1.5 - 9.9	**0.006**	2.1	0.8-5.9	0.145	0.4	0.04 - 3.7	0.406	4.4	0.9-21.3	0.062
Treatment												
(C)RT vs Sx ± (C)RT	0.6	0.2 - 1.4	0.239									
TLR 5												
2-3 vs 0-1	4.2	1.4 - 12.8	**0.011**	2.5	0.8 - 8.1	0.1 9	14.2	1.3 - 151.1	**0.028**	0.9	0.2-3.6	0.906
HPV status												
HPV-negative vs HPV-positive	3.6	1.3 - 9.6	**0.010**	1.3	0.4 - 4.	0.649						

T class (primary tumor size), N class (presence of regional lymph node metastasis), Sx + (C)RT (surgery + chemoradiotherapy), C(RT) (chemoradiotherapy) TLR5 (toll-like receptor 5 immunoexpression), HPV-positive (p16- and HPV-DNA-positive OPSCC), HPV-negative (p16-negative and HPV-DNA-positive or –negative OPSCC, and p16-positive and HPV-DNA negative OPSCC). Statistically significant *P* values bolded.

Figure 1. Disease-specific survival (DSS) curves for human papilloma virus (HPV) -positive and HPV-negative tumors according to toll-like receptor (TLR) 5 expression

Figure 2. Flagellin-induced changes in NF-κB signaling cascade in cell lines (HaCat, UT-SCC-65 and UT-SCC-69). Relative amount of IκB (A); relative amount of p-IκB (B); and relative p-IκB/ IκB (C) expression presented as function of time after TLR5 stimulation with flagellin. Nuclear marker DRAQ-5 served for normalization. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Elevated TLR5 expression *in vivo* and loss of NF-κB activation via TLR5 *in vitro* detected in HPV-negative oropharyngeal squamous cell carcinoma

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- High TLR5 expression was associated with poor disease-specific survival in HPV-positive OPSCC, which typically shows low TLR5 immunoexpression.
- High TLR5 immunoexpression was more common in HPV-negative OPSCC, known for its less-favorable prognosis.
- *In vitro* stimulation of TLR5 with flagellin activated the NF-κB cascade in HPV-positive OPSCC cells and in HaCat cells, whereas no activation of the NF-κB cascade in HPV-negative OPSCC cells occurred.
- Our results suggest that elevated TLR5 immunoexpression may be related to reduced NF-κB activity in HPV-negative OPSCC. The possible prognosis-worsening mechanisms among these high-risk OPSCC patients however, require further evaluation.